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Genes predisposing to autoimmunity augment constitutive major histocompatibility complex class II-associated presentation of the self-antigen IgG2a^b *in vivo*

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SUMMARY

The self-antigen IgG2a^b is poorly presented to a γ2a^b 435–451-reactive I-A^d-restricted T-cell hybridoma unless available in high concentrations or targeted to Fcγ- or complement receptors. Environmental factors, probably the extent of microbial challenge, profoundly influence the constitutive γ2a^b/I-A^d presentation in IgC^b_H, H-2^d mice. Here we report also a strong genetic impact. Constitutive presentation was highly efficient in spleen and thymus of (NZB $\times$ BXSB)F$_1$ mice, which inherit a predisposition to develop lupus. Presentation correlated with disease progression and the serum levels of IgG2a^b and IgG2a^b complement factor 3 complexes. The finding that constitutive presentation was by far most efficient in males indicated that it was augmented by the Y chromosome-linked autoimmune acceleration *Yaa* gene. In line with previous data for healthy mice, constitutive γ2a^b/I-A^d presentation was most pronounced in the adherent spleen cell fraction and improved by further enrichment for dendritic cells. Notably, however, whereas in normal mice the γ2a^b determinant was undetectable on B cells lacking surface IgG2a^b, such B cells contributed considerably to constitutive presentation in (NZB $\times$ BXSB)F$_1$ hybrids. Presumably this resulted from complement receptor-mediated internalization of IgG2a^b-containing immune complexes formed in lupus. These data add to the evidence that B cells with self-reactive receptors, known to exist in the mature repertoire, may present non-cognate foreign antigen to anti-foreign helper T lymphocytes and thus differentiate into autoantibody-secreting cells, and might likewise account for the polyclonal B-cell activation characteristic of several autoimmune syndromes.

INTRODUCTION

The mature T helper (Th) cell repertoire includes clones specific for syngeneic immunoglobulin variable (V) region peptide/major histocompatibility (MHC) class II complexes.[1–4] By recognizing the clonotypic immunoglobulin of B-cell neoplasms as a tumour-specific antigen,[5] Th cells can confer tumour resistance.[6] This concept may find clinical applications, as immunization with the tumour immunoglobulin eradicated an established murine lymphoma[7] and conferred prolonged remission and tumour regression in non-Hodgkin's lymphoma patients.[8] Th cell recognition of immunoglobulin may also be involved in immune response regulation and in the pathogenesis of autoimmune diseases. Thus, I-A^d-restricted Th clones specific for the immunoglobulin G2a^b (IgG2a^b) heavy (H) chain allopeptide γ2a^b 435–451 selectively suppressed B cells that presented their intrinsic IgG2a^b *in vivo,*[9,10] and the γ2a^b 435–451 determinant mimics a corneal autoantigen implicated in herpes simplex virus (HSV)-induced autoimmune stromal keratitis[11] as well as the UL6-299–314 epitope of a keratitogenic HSV-type 1 strain.[12] However, native IgG2a^b is a surprisingly weak antigen for I-A^d-restricted T cells, as 700 IgG2a^b molecules (1400 epitopes) were required to generate the antigenic equivalent of a single γ2a^b peptide.[13] Internalization by antigen-presenting cells (APC) was limiting, as targeting to FcγR or complement receptors (CR) strikingly augmented T-cell responses. Notably, the γ2a^b 435–451/I-A^d reactive T-cell hybridoma, B5, responds to APC from IgC^b_H, I-A^d mice in the absence of exogenous IgG2a^b and should represent a useful, quantitative probe for analysing constitutive γ2a^b/I-A^d presentation *in vivo*. Thus, B5 is insensitive to B7-mediated

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Abbreviations: APC, antigen-presenting cell; CR, complement receptor; H chain, immunoglobulin heavy chain; [^{3}H]Tdr, tritiated thymidine; HSV, herpes simplex virus; mAb, monoclonal antibody; sIg, surface immunoglobulin; SLE, systemic lupus erythematosus; SPF, specific pathogen-free; Th, T helper; V region, immunoglobulin variable region; *Yaa* gene, Y chromosome-linked autoimmune acceleration gene.

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signals,[14] the interactions of the $\gamma 2a^b$ determinant with the B5 antigen receptor and I-A^d have been characterized in detail,[10,15,16] and the B5-defined epitope is among the predominant determinants generated by processing of $IgG2a^b$ (refs 10,11; K. Bartnes, unpublished). Furthermore, constitutive presentation is independent of maturation of APC and intercellular antigen transfer *in vitro*, as cells that were paraformaldehyde fixed immediately after preparation were stimulatory, and a co-culture of spleen cells from BALB/c (I-A^d, IgC_H^a,) and BXSB (I-A^b, IgC_H^b,) failed to stimulate B5 (K. Bartnes, unpublished). Confirming the poor antigenicity of native $IgG2a^b$ *in vitro*, constitutive presentation was weak or absent in specific pathogen-free (SPF) healthy animals, which had low levels of circulating $IgG2a^b$–C3 complexes and not more than a few hundred μg/ml of serum $IgG2a^b$.[13,14] In contrast, spleen cells from mice reared under less rigorous microbial control regularly elicited strong B5 hybridoma responses,[9,13] demonstrating a profound influence of environmental factors on the constitutive presentation of the $IgG2a^b$ self-antigen. To the extent that these findings apply to IgG in general, analyses of constitutive presentation of $IgG2a^b$ to the $\gamma 2a^b$/I-A^d-reactive T-cell hybridoma, B5, may be useful in elucidating the factors that determine IgG peptide presentation and thus provide information relevant both in the immunopathogenesis of HSV-induced autoimmune keratitis and for designing clonotypic immunoglobulin vaccines against lymphomas. It is also relevant in regard to the problem of neutralization of therapeutic antibodies (Ab) by Th-dependent anti-immunoglobulin Ab.

The weak and infrequent constitutive presentation in normal, SPF IgC_H^b, H-2^d mice hampered further development of this experimental model. Accordingly, because immune complex formation strongly promotes the antigenicity of $IgG2a^b$,[13] we examined (NZB × BXSB)F_1 mice ($IgC_H^{e/b}$, H-$2^{d/b}$) that produce $IgG2a^b$ immune complexes, thus reflecting an inherited predisposition to systemic lupus erythematosus (SLE).[17] (NZB × BXSB)F_1 spleen and thymus cells efficiently presented $\gamma 2a^b$/I-A^d concomitantly with the onset of SLE, demonstrating a profound genetic influence on constitutive presentation.

MATERIALS AND METHODS

Mice

NZB and BXSB breeders were obtained from Harlan Olac Ltd (Bicester, UK) and The Jackson Laboratory (Bar Harbor, ME), respectively. (NZB × BXSB)F_1 hybrids were bred and kept under SPF conditions.

Cells

The T-cell hybridoma, B5, was derived from a $CD4^+$ T helper 1 (Th1) clone originating in a BALB/c mouse immunized with allogeneic IgG2a ($IgG2a^b$)[9] and recognizes the $\gamma 2a^b$ 435–451 (Kabat numbering) H-chain peptide/I-A^d complex.[10] Splenic single-cell suspensions were prepared using standard procedures. Adherent cells were isolated essentially as described previously.[18] Briefly, spleen cells were incubated on plastic Petri dishes in RPMI-1640 (Gibco Ltd, Paisley, Strathclyde, UK) supplemented with synthetic serum replacement (SSR-3) (Medi-Cult; GEA-Biotech, Hvidovre, Denmark) for 2 hr at 37°. Following removal of non-adherent cells by washing in warm phosphate-buffered saline (PBS), adherent cells were dislodged using a cell scraper (Costar Corp., Cambridge, MA) after incubation for 15 min in ice-cold PBS containing 20 mM EDTA. Non-adherent cells were isolated by passing the cell suspensions through Sephadex G10 (Pharmacia, Uppsala, Sweden) columns at 37°. Dendritic cell enrichment was performed as described previously.[19] Briefly, spleens teased with hypodermic needles were incubated with collagenase (2·4 mg/ml) and DNase (0·1 mg/ml) (Sigma, St. Louis, MO) for 30 min at 37°, centrifuged to equilibrium on 60% Percoll (Pharmacia), and the secondary non-adherent cells from the buoyant fraction were collected. These contained ≈45% dendritic cells, as assessed by fluorocytometric analysis of samples stained with the CD11c-specific monoclonal antibody

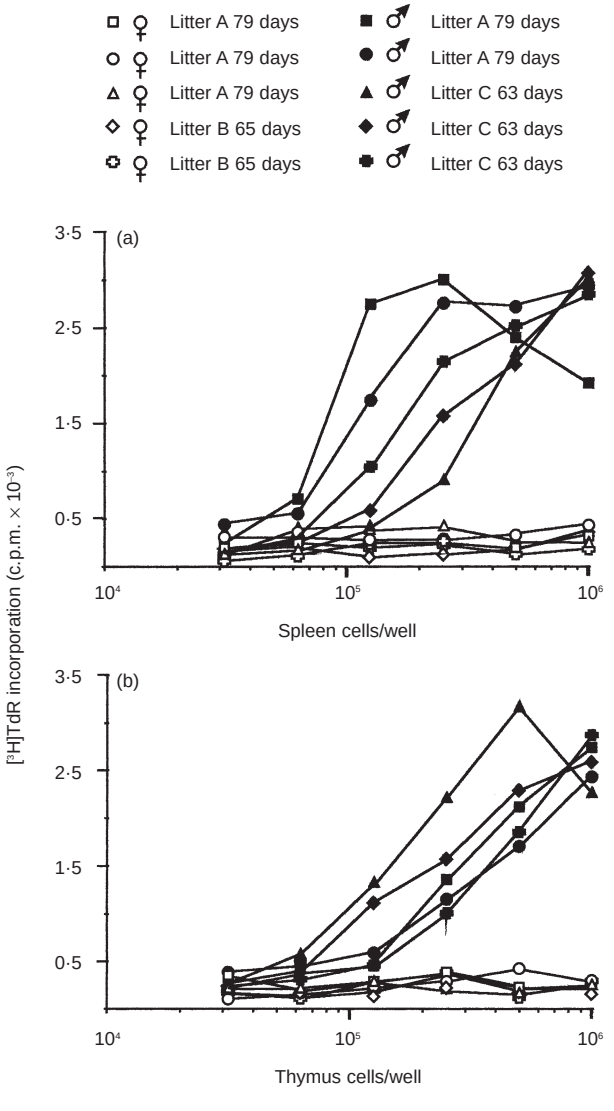

Figure 1. Capacity of serially diluted spleen (a) and thymus (b) cells from individual 9–11-week-old (NZB × BXSB)F_1 mice to stimulate the $\gamma 2a^b$ peptide/I-A^d-reactive T-cell hybridoma B5 in the absence of exogenous antigen. The interleukin-2 (IL-2) activity, as measured by [^{3}H]thymidine ([^{3}H]Tdr) incorporation by HT-2 cells in 5% 24-hr culture supernatant, is shown (mean of triplicates). c.p.m., counts per minute.

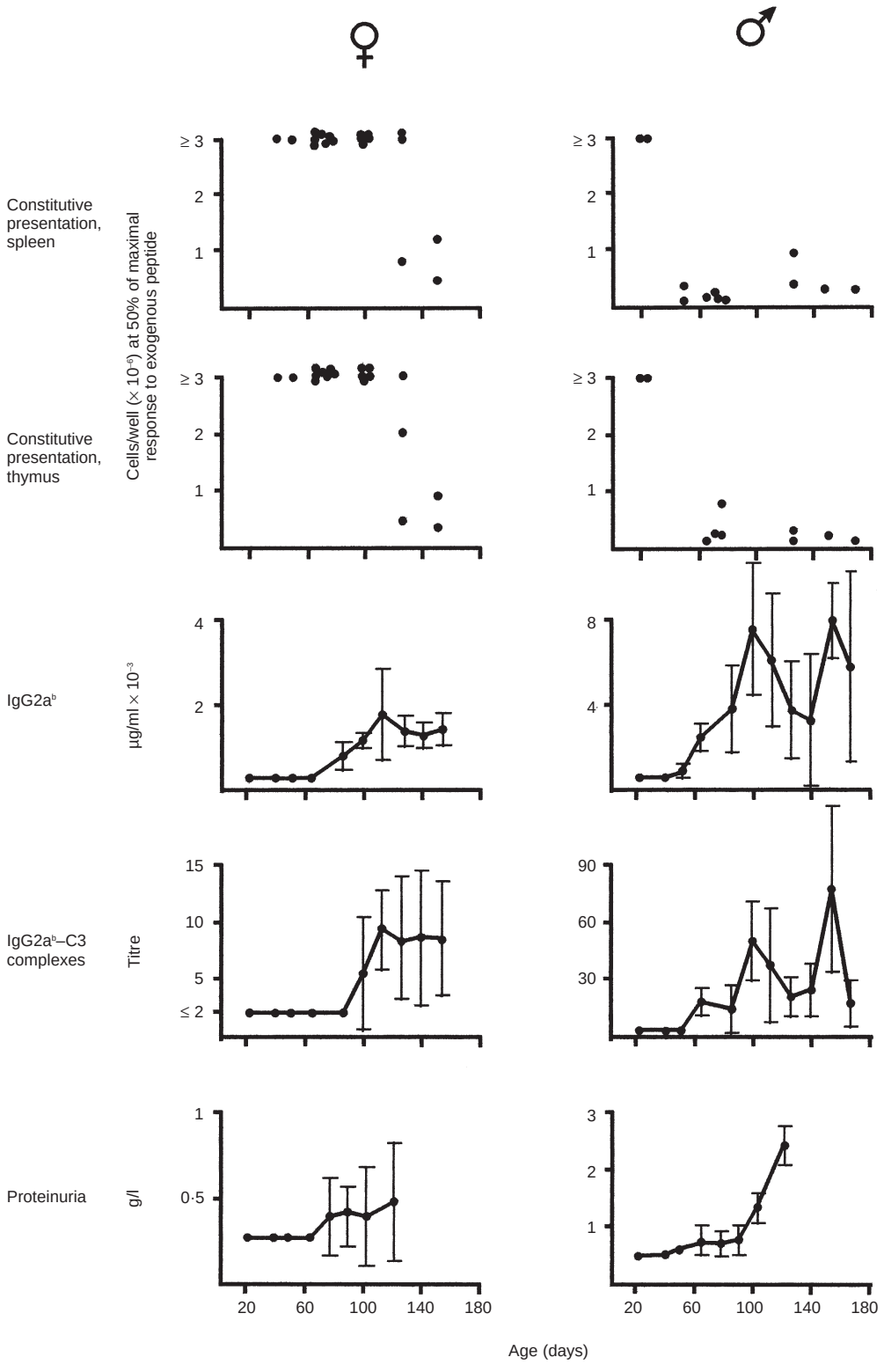

Figure 2. Constitutive $\gamma 2a^b$/I-A^d presentation by (NZB × BXSB)F_1 spleen and thymus cells, serum immunoglobulin G2a^b (IgG2a^b), serum IgG2a^b–C3 and proteinuria as a function of age and gender. The four upper panels display individual constitutive presentation capacity values for a cohort of four litters (19 females, 12 males). Constitutive presentation is expressed as the number of spleen or thymus cells/well ($\times 10^{-6}$) that corresponded to 50% of maximal hybridoma B5 response to the exogenously supplied antigenic peptide $\gamma 2a^b$ 435–451. The six lower panels display the serum levels of IgG2a^b and IgG2a^b–C3 and urine albumin content (mean ± SD) for the part of the cohort remaining as time progressed.

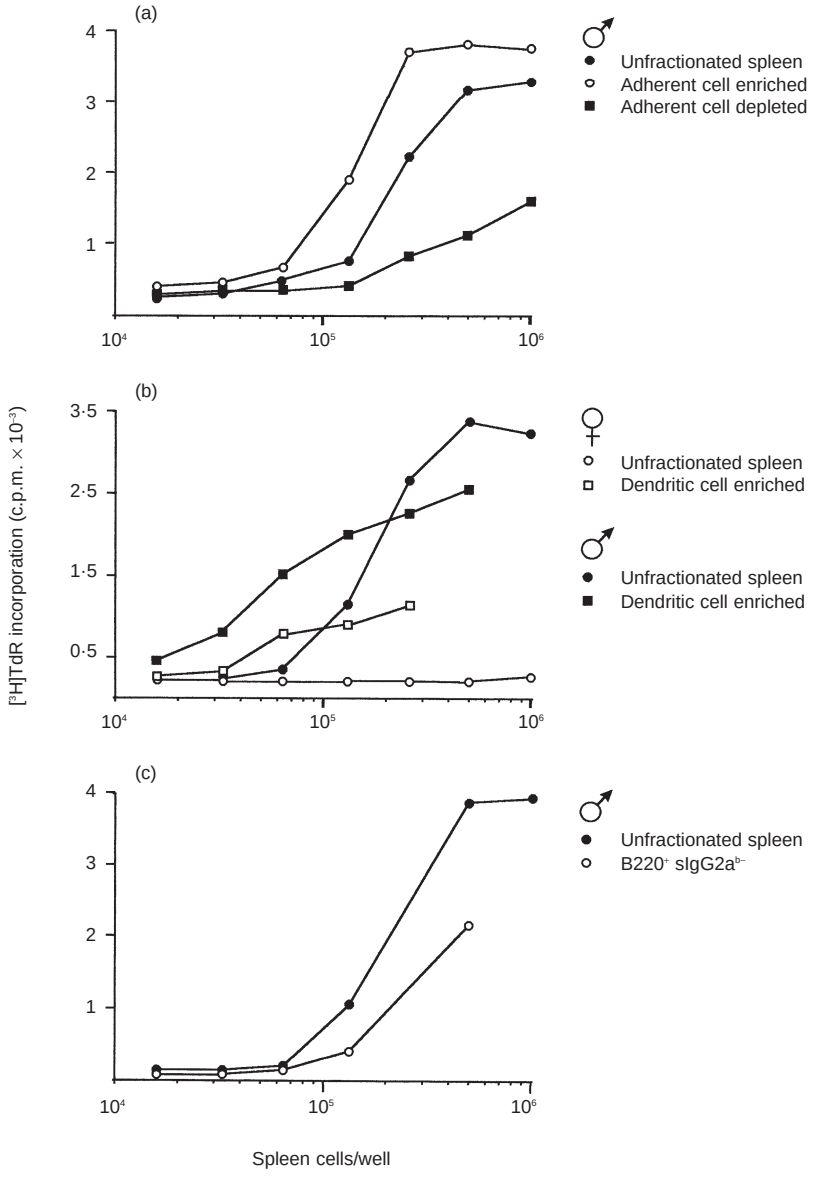

Figure 3. Constitutive immunoglobulin G2a^b (IgG2a^b)/I-A^d presentation by various (NZB × BXSB)F$_1$ spleen cell populations. Three separate experiments are shown. (a) Enrichment for plastic-adherent and depletion of Sephadex G10-adherent cells from a pool of spleen cells from three 10-week-old male hybrids was performed as described in the Materials and methods. (b) Spleen cells from individual mice from the experiment shown in Fig. 1 were pooled and enriched for dendritic cells as described previously.[16] (c) B220$^+$ surface (s)IgG2a^{b-} spleen cells from a pool of four mice were isolated by fluorescence-activated cell sorter (FACS) analysis, as described in the Materials and methods. Notably, irradiation (2000 rads) prior to labelling ensured that the responses did not result from anti-sIgG2a^b Ab-induced γ2a^b/I-A^d presentation.[14] The various cell preparations were serially diluted and tested for their capacity to elicit B5 hybridoma interleukin-2 (IL-2) secretion. Values shown represent [^{3}H]thymidine ([^{3}H]Tdr) incorporation by HT-2 cells in 5% 24-hr culture supernatant (mean of triplicates).

(mAb) N418, and $<50\%$ B220$^+$ cells (results not shown). Thymic single-cell suspensions were obtained by incubating the lobes (teased with bent hypodermic needles) in collagenase and DNase followed by gentle pipetting, removal of debris by sedimentation at 1 *g*, and washing. Cultures were maintained in RPMI-1640 supplemented with 10% heat-inactivated bovine serum (HyClone, Logan, UT), 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 12 mM HEPES and 0·05 mM 2-mercaptoethanol (2-ME), and incubated at 37° in humidified air containing 5% CO_2.

Antigen-presentation assay

Spleen and thymus cells to be assessed for constitutive presentation capacity were serially diluted and cultured with 5×10^4 B5 hybridoma cells per well in the presence of antigen or medium alone, and hybridoma growth inhibition[20] or interleukin-2 (IL-2) secretion was assessed. IL-2 activity in pooled triplicates of 24-hr hybridoma culture supernatants was determined as described previously,[9] based on proliferation of HT-2 cells. Values shown represent [^{3}H]thymidine ([^{3}H]Tdr) incorporation determined using a Matrix^{96} Direct β Counter

Table 1. Correlation between constitutive presentation capacity and age, serum IgG2a^b, serum IgG2a^b–C3 and proteinuria at the time of death

Source of APC	Parameter	ρ	95% CI	P-value
Spleen	Age	0·31	−0·04–0·59	0·0840
	Serum IgG2a^b	0·60	0·31–0·78	0·0003
	Serum IgG2a^b–C3	0·54	0·24–0·75	0·0014
	Proteinuria	0·61	0·33–0·79	0·0002
Thymus	Age	0·59	0·30–0·79	0·0005
	Serum IgG2a^b	0·68	0·42–0·84	0·0001
	Serum IgG2a^b–C3	0·75	0·54–0·88	0·0001
	Proteinuria	0·52	0·20–0·74	0·0030

The calculations for spleen and thymus were based on individual values from 32 and 30 (NZB × BXSB)F_1 hybrid mice of both genders, respectively, including those shown in Fig. 1.

(Packard Instrument Company, Meriden, CT). Standard error of the mean (SEM) values were below 15%. To ensure that the sensitivity of the antigen-presentation assay was similar among different experiments, a challenge with titrated peptide γ2a^b 435–451 and irradiated (2000 rads) BALB/c spleen cells as APC was always included.

Enzyme-linked immunosorbent assay (ELISA)

For IgG2a^b quantification, polystyrene microtitre wells (Immunoplate Maxisorp; Nunc, Roskilde, Denmark) were coated with the IgG2a^b-specific IgG1 mAb G12–47/30[21] (10 μg/ml) in PBS followed by saturation of the plastic with 0·5% bovine serum albumin (BSA). Serially diluted samples were incubated overnight followed by sequential 2-hr incubations with biotin-labelled goat anti-mouse IgG2a (Southern Biotechnology Associates Inc., Birmingham, AL) (0·7 μg/ml) and alkaline phosphatase-conjugated streptavidin (Zymed, San Francisco, CA) (1 μg/ml). All steps were performed at 4°. The IgG2a^b concentrations were determined by comparing sample titration curves with that of a standard, purified IgG2a^b, the preparation of which had been quantified spectrophotometrically. For quantification of C3–IgG2a^b complexes, wells were coated with goat F(ab′)$_2$ anti-C3 (1 : 50 dilution; Cappel Laboratories, Cochranville, PA), at pH 9. Following saturation of the plastic with BSA, serially diluted samples were incubated for 2 hr at 37° followed by sequential incubation at 4° with G12–47/30 (1 μg/ml, overnight), biotin-labelled goat anti-mouse IgG1 (0·2 μg/ml, 2 hr; Southern Biotechnology Associates Inc.) and alkaline phosphatase-conjugated streptavidin (0·2 μg/ml, 2 hr). A reference serum from a BXSB (IgC$_H^b$) mouse with advanced immune complex disease was included as an internal standard in every analysis. In both assays, samples and conjugates were diluted in 0·2% PBSA containing 0·05% Tween-20, which was also used for washing the plates between each step. The enzyme reaction was developed using *p*-nitrophenyl phosphate (Sigma) as substrate. Absorbance at 405 nm was determined using a Titertek Multiscan spectrophotometer (Flow Laboratories, Titertek Instruments Inc, Huntsville, AL).

Fluorescence-activated cell sorting

A fluorescence-activated cell sorter (FACStar; Becton-Dickinson, Mountain View, CA) was used to aseptically isolate B220$^+$ IgG2a^{b-} cells from spleen-cell suspensions sequentially incubated with the biotin-conjugated B220-specific mAb RA3–6B2 (Pharmingen, San Diego, CA) (10 μg/ml) and alexa-G12–46/30 (10 μg/ml) combined with r-phycoerythrin–streptavidin. In order to block FcγR binding, incubation with the mAb was performed in the presence of supernatant from the 2.4G2 hybridoma (American Type Culture Collection, Rockville, MD) producing FcγRII/III-specific mAb.

Miscellaneous

Urine protein content was determined using the Combur^{10}-Test (Boehringer Mannheim, Lewes, UK). Conjugation of alexa to G12–46/30 and a control murine IgG1 was performed using the Alexa™ 488 Protein Labelling Kit (Molecular Probes Inc., Eugene, OR).

Statistical analysis

Spearman's correlation coefficient (ρ), confidence intervals and *P*-values were calculated using the SAS System software (SAS Institute Inc., Cary, NC).

RESULTS

Profound genetic influence on constitutive γ2a^b peptide/I-A^d-presentation

(NZB × BXSB)F_1 hybrid mice spontaneously develop an autoimmune syndrome that closely resembles SLE in humans and is characterized by hypergammaglobulinaemia, autoantibody production and immune-complex glomerulonephritis.[17] As a result of the Y chromosome-linked autoimmune-accelerating *Yaa* gene, the disease commences earlier and progresses more rapidly in F_1 hybrid males (50% cumulative mortality rates caused by glomerulonephritis of F_1 male and female mice: 7 and 9 months, respectively).[22] Intriguingly, whereas spleen cells from the vast majority, i.e. 82% of normal IgC$_H^b$, H-2^d mice (B10.D2, CB.17 and others) failed to elicit any response by the γ2a^b-reactive I-A^d-restricted T-cell hybridoma B5,[13] all adult (NZB × BXSB)F_1 hybrid males tested simultaneously and reared under the same SPF conditions exhibited strong constitutive γ2a^b presentation (Figs 1 and 2). Notably, B5 responses were not biased by cross-reactivity with the IgG2a of the e-allotype encoded on the NZB-derived haplotype (data not shown), consistent with non-conservative amino acid replacements corresponding to γ2a^b residues 444 and 446 that are critical for B5 recognition.[15,23] At age 9–11 weeks, the capacity to detectably present γ2a^b/I-A^d *in vivo* segregated absolutely with the Y-chromosome, as presentation was undetectable in all female littermates (Fig. 1). At advanced age, following the development of SLE manifestations such as hypergammaglobulinaemia, increased circulating immune complex levels and glomerulonephritis (detected as proteinuria), female mice also acquired constitutive presentation capacity (Fig. 2). Constitutive presentation correlated significantly with age, proteinuria and serum levels of IgG2a^b and IgG2a^b–C3 complexes (Table 1). Thus, at 14 weeks of age, i.e. when all males (but none of the females) detectably presented γ2a^b/I-A^d constitutively, the average serum IgG2a^b concentrations were 1200 and 7600 μg/ml, IgG2a^b-C3 titres were 6 and 50, and urine protein content was 0·4 and 1·0 g/l for F_1 females

and males, respectively (Fig. 2). In contrast, the corresponding values for normal IgC_H^b, H-2^d mice (B10.D2 and CB.17) were 40 μg/ml, <2[13] and $<0{\cdot}3$ g/l (results not shown). The data for the female mice demonstrate that SLE-predisposing genes profoundly augment constitutive $\gamma2a^b$/I-A^d-presentation capacity. Moreover, the striking superiority of APC from male mice implicates the *Yaa* gene as a genetic element with strong impact on the capacity to constitutively present $\gamma2a^b$/I-A^d.

Identification of the IgG2a^b-presenting cell types in spleen

Constitutive presentation by *ex vivo* (NZB × BXSB)F_1 spleen cells was strongest in the plastic-adherent fraction (Fig. 3a) and was improved by further enrichment for dendritic cells (Fig. 3b). We previously established that surface (s)IgG2a^b B lymphocytes from normal mice present their intrinsic immunoglobulin *in vivo*.[9] As circulating IgG2a^b–C3 complexes were found in the SLE-affected mice, but rarely in normal mice, and because B cells efficiently present IgG2a^b targeted to CR,[13] we reasoned that B cells without sIgG2a^b may also present $\gamma2a^b$/I-A^d. Consistent with this idea, FACS-sorted B220$^+$ sIgG2a^{b-} spleen cells from SLE-affected (NZB × BXSB)F_1 mice elicited substantial B5 responses (Fig. 3c).

DISCUSSION

We demonstrate here that efficient constitutive presentation of $\gamma2a^b$/I-A^d in the SLE-prone (NZB × BXSB)F_1 hybrid correlates with the development of glomerulonephritis and with the serum levels of IgG2a^b and circulating IgG2a^b–C3 complexes. Whereas previous studies showed that environmental factors strongly influenced constitutive presentation capacity,[13] the current data also reveal a strong genetic impact. Hence, the constitutive presentation exhibited by lupus-affected female (NZB × BXSB)F_1 mice demonstrates that SLE-susceptibility genes substantially augment $\gamma2a^b$ peptide/I-A^d expression. Moreover, the superior presentation capacity by male hybrids identifies an additional Y chromosome-linked effect, probably conferred by the disease-accelerating *Yaa* gene.

The efficient constitutive presentation by SLE mice probably reflects their innate production of IgG2a-containing immune complexes.[17] Thus, aggregated IgG2a^b is >200-fold more potently presented by macrophages than native IgG2a^b owing to internalization via FcγRII/III.[13] Notably, whereas adherent, and particularly dendritic, cells are the predominating splenic $\gamma2a^b$-presenting APC in healthy[13] and lupus mice (this study), and also sIgG2a^b B cells constitutively present their intrinsic immunoglobulin to CD4$^+$ T cells,[9] efficient presentation by non-sIgG2a^b B cells is a distinguishing feature of SLE-affected mice. Thus, B cells isolated from the spleens of normal strains were invariably unstimulatory to the $\gamma2a^b$/I-A^d-reactive T-cell hybridoma (ref. 14; K. Bartnes, unpublished). The strong correlation between constitutive presentation and serum IgG2a^b–C3 levels, combined with the previously reported poor capacity of native IgG2a^b to sensitize B cells for B5 recognition,[13] suggests that non-sIgG2a^b B cells constitutively present $\gamma2a^b$ as a result of CR-mediated endocytosis of IgG2a^b that has fixed complement. In support of this contention, targeting IgG2a^b to CR potently endowed the B-cell lymphoma A20 with $\gamma2a^b$/I-A^d-presentation capability.[13] As the *Yaa* mutation is probably expressed in B cells,[24] the possibility has to be considered that presentation by non-sIgG2a^b B cells was the result of a generally improved antigen-presentation capacity conferred by the *Yaa* gene. However, the finding that non-sIgG2a^b B cells from not only male but also female hybrids exhibited constitutive presentation (K. Bartnes, unpublished) excluded a requirement of *Yaa* expression for $\gamma2a^b$ presentation by non-sIgG2a^b B cells.

B-cell clonal deletion is an incomplete safeguard against humoral autoimmunity because the normal, mature B-cell repertoire contains clones with self-antigen reactive sIg receptors.[25] Therefore, tolerance at the Th level appears to be essential in order to avoid inappropriate B-cell activation. Indiscriminate antigen presentation by B cells, i.e. efficient ingestion of an antigen capable of bypassing the censoring step conferred by selective recognition by the B-cell antigen receptor, might initiate activation of forbidden B-cell clones by anti-foreign Th cells. In line with this notion, B cells are inefficient at fluid-phase pinocytosis[26] and their FcγR isoform, FcγRIIb1, is endocytosis incompetent.[27] The findings that antigen can be targeted to the compartment(s) of antigen processing and class II MHC peptide loading by B-cell CR1[13] and CR2,[28] combined with the evidence that CR-mediated internalization makes non-sIgG2a^b B lymphocytes present non-cognate $\gamma2a^b$ antigen *in vivo*, is therefore intriguing. It opens the possibility that autoantibody production results from interactions between anti-foreign Th cells and anti-self B lymphocytes presenting non-cognate foreign antigen internalized as complement-coated microbial proteins. This type of Th–B-cell interaction might also account for the polyclonal B-cell activation characteristic of murine SLE and several autoimmune diseases in humans.[29]

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